Defining the Region of Bacillus subtilis SpoIIIJ That Is Essential for Its Sporulation-Specific Function

Geng, Yanping; Keyzer, Jeanine de; Scheffers, Dirk-Jan; Driessen, Arnold J.M.

Published in:
Journal of Bacteriology

DOI:
10.1128/JB.01084-13

JB
Journals.ASM.org

Defining the Region of *Bacillus subtilis* SpoIIIJ That Is Essential for Its Sporulation-Specific Function

Yanping Geng, Jeanine de Keyzer, Dirk-Jan Scheffers, Arnold J. M. Driessen

Molecular Microbiology, Groningen Biomolecular Sciences and Biotechnology Institute and Zernike Institute for Advanced Materials, Groningen, The Netherlands

Proteins of the YidC/OxaI/Alb3 family play a crucial role in the insertion, folding, and/or assembly of membrane proteins in prokaryotes and eukaryotes. *Bacillus subtilis* has two YidC-like proteins, denoted SpoIIIJ and YqjG. SpoIIIJ and YqjG are largely exchangeable in function, but SpoIIIJ has a unique role in sporulation, while YqjG stimulates competence development. To obtain more insight into the regions important for the sporulation specificity of SpoIIIJ, a series of SpoIIIJ/YqjG chimeras was constructed. These chimeras were tested for functionality during vegetative growth and for their ability to complement the sporulation defect of a *spoIIIJ* deletion strain. The data suggest an important role for the domain comprising transmembrane segment 2 (TMS2) and its flanking loops in sporulation specificity, with lesser contributions to specificity by TMS1 and TMS3.

In prokaryotes, most membrane proteins are delivered to and integrated into the membrane via the signal recognition particle (SRP) and the Sec translocon. However, a subset of membrane proteins requires the aid of the YidC/OxaI/Alb3 protein family (1–4). This protein family is composed of evolutionarily conserved integral membrane proteins, which are present in all domains of life and play an important role in the insertion, integration, folding, and/or assembly of membrane proteins (1–5). Members of the YidC/OxaI/Alb3 protein family exhibit rather low primary sequence similarity but share a conserved hydrophobic core region composed of five transmembrane segments (TMSs) linked by short hydrophilic loops.

Bacillus subtilis contains two YidC paralogues, SpoIIIJ and YqjG (34% identical and 46% similar), which are expressed constitutively during vegetative growth and sporulation. It has been suggested that SpoIIIJ and YqjG are implicated in the posttranslocational stages of protein secretion (6), but recent data from our laboratory demonstrate that SpoIIIJ and YqjG fulfill a role in membrane protein insertion, similar to YidC of *Escherichia coli* (4). YqjG and SpoIIIJ are largely exchangeable in function, and either protein is sufficient for vegetative growth, with an upregulation of the level of YqjG when the SpoIIIJ activity is absent or decreased (4, 6–8). During *B. subtilis* development, each protein has a specific role. YqjG has a specific function in enhancing competence development, which cannot be complemented by SpoIIIJ (9). However, YqjG is not essential for competence development. On the other hand, SpoIIIJ is essential during sporulation and is required for the activation of late-sporulation sigma factor G. In the absence of SpoIIIJ, sporulation is arrested at stage III (7). Expression of SpoIIIJ in the prespore alone is sufficient for the activation of σ^G soon after the completion of engulfment (10). Fluorescence microscopy studies with fusion proteins using the green fluorescent protein reporter indicate that SpoIIIJ and YqjG are both distributed throughout the membrane and accumulate at septa during the onset of the sporulation (11). SpoIIIJ interacts with the sporulation-specific membrane protein SpoIIIAE, and SpoIIIAE expression in the absence of SpoIIIJ is lethal, although SpoIIIAE is still inserted into the membrane in the absence of SpoIIIJ (12). These results suggest that SpoIIIJ assists in the correct folding of SpoIIIAE, but as SpoIIIAE also interacts with YqjG and is still inserted into the membrane in the absence of SpoIIIJ, the exact mode of action still remains to be resolved (12). Recent proteomics studies reveal that the absence of SpoIIIJ results in the increased expression of proteins involved in the late stages of sporulation, e.g., SpoIIIAH (9), again indicating a role for SpoIIIJ in the assembly or correct folding of these proteins after insertion into the membrane.

Until now, little has been known about the features of SpoIIIJ that are required for its sporulation-specific function and that distinguish it from YqjG. To address this question, we constructed a set of SpoIIIJ-YqjG chimeric proteins by exchanging TMSs from the conserved 5-TMS hydrophobic core and tested their ability to complement the sporulation defect of a SpoIIIJ deletion strain. Our data suggest that TMS1, TMS3, and in particular TMS2 and its hydophilic flanking loops play a critical role in the sporulation specificity of SpoIIIJ.

MATERIALS AND METHODS

Strains and plasmids. All strains used in the study are listed in Table 1. *E. coli* DH5α was used as a cloning host. *B. subtilis* 168 was transformed as described previously (13). The SpoIIIJ-YqjG and YqjG-SpoIIIJ chimers were constructed by using overlapping PCR with the nucleotide primers shown in Table S1 in the supplemental material and chromosomal DNA of *B. subtilis* 168 as the template. For the $S_{TMS1345}$-$Y_{TMS2syn}$ mutant, a synthetic *spoIIIJ* gene was ordered, in which the sequence encoding TMS2 (amino acids 131 to 151) was swapped with the sequence encoding TMS2 of YqjG (amino acids 139 to 159) (GeneArt, Life Technologies) and amplified with primers chimera456fw and chimera123rev. The resulting chimeric genes were doubly digested with NheI and SphI and cloned between the *amyE*-front and *amyE*-back homologous fragments of NheI/SphI-digested pDR111 (a gift of David Rudner), yielding the respective plasmids pDRchimer1 to pDRchimer8 (Table 1). Control constructs containing full-length *spoIIIJ* and *yqjG* were constructed in the same way.

Received 12 September 2013 Accepted 13 January 2014
Published ahead of print 17 January 2014
Address correspondence to Arnold J. M. Driessen, a.j.m.driessen@rug.nl.
Supplemental material for this article may be found at http://dx.doi.org/10.1128/JB.01084-13.

doi:10.1128/JB.01084-13

TABLE 1 List of strains and plasmids

Plasmid or strain	Relevant property(ies)	Source or reference
Plasmids		
pDR111	$P_{hyper\text{-}spank}$; Spc^r Amp^r; expression vector for *B. subtilis*	Gift of David Rudner
pDRspoIIIJ	Spc^r; $P_{hyper\text{-}spank}$-*spoIIIJ* fusion	This study
pDRspoY	Spc^r; containing *spoIIIJ* with TMS2 replaced by *yqjG* TMS2	This study
pDRyqjS	Spc^r; containing *yqjG* with TMS2 replaced by *spoIIIJ* TMS2	This study
pDRyqjG	Spc^r; $P_{hyper\text{-}spank}$-*yqjG* fusion	This study
pDRchimer1	Spc^r; $P_{hyper\text{-}spank}$-Y_{TMS1}-$S_{TMS2345}$ fusion	This study
pDRchimer2	Spc^r; $P_{hyper\text{-}spank}$-Y_{TMS12}-S_{TMS345} fusion	This study
pDRchimer3	Spc^r; $P_{hyper\text{-}spank}$-Y_{TMS123}-S_{TMS456} fusion	This study
pDRchimer4	Spc^r; $P_{hyper\text{-}spank}$-S_{TMS1}-$Y_{TMS2345}$ fusion	This study
pDRchimer5	Spc^r; $P_{hyper\text{-}spank}$-S_{TMS12}-Y_{TMS345} fusion	This study
pDRchimer6	Spc^r; $P_{hyper\text{-}spank}$-S_{TMS123}-Y_{TMS456} fusion	This study
pDRchimer7	Spc^r; $P_{hyper\text{-}spank}$-Y_{P1}-$S_{TMS12345}$ fusion	This study
pDRchimer8	Spc^r; $P_{hyper\text{-}spank}$-S_{P1}-$Y_{TMS12345}$ fusion	This study
pNZ8901	SURE expression vector for *B. subtilis*; P*spaS*$_{mut}$; Cm^r	14
pNZspoIIIJ	pNZ8901 containing *B. subtilis spoIIIJ*	This study
pNZspoIIIJhis	pNZ8901 containing an N-terminal His_6 tag fusion of *spoIIIJ*	4
pNZspoY$_{TMS2}$	pNZ8901 containing the chimeric $S_{TMS1345}$-Y_{TMS2} gene	This study
pNZyqjG	pNZ8901 containing *B. subtilis yqjG*	This study
pNZyqjGhis	pNZ8901 containing an N-terminal His_6 tag fusion of *yqjG*	4
pNZyqjS$_{TMS2}$	pNZ8901 containing the chimeric $Y_{TMS1345}$-S_{TMS2} gene	This study
pNZspoY$_{TMS2syn}$	pNZ8901 containing the chimeric $S_{TMS1345}$-$Y_{TMS2syn}$ gene	This study
pNZchimer1	Cm^r; P*spaS*$_{mut}$-Y_{TMS1}-$S_{TMS2345}$ fusion	This study
pNZchimer2	Cm^r; P*spaS*$_{mut}$-Y_{TMS12}-S_{TMS345} fusion	This study
pNZchimer3	Cm^r; P*spaS*$_{mut}$-Y_{TMS123}-S_{TMS45} fusion	This study
pNZchimer4	Cm^r; P*spaS*$_{mut}$-S_{TMS1}-$Y_{TMS2345}$ fusion	This study
pNZchimer5	Cm^r; P*spaS*$_{mut}$-S_{TMS12}-Y_{TMS345} fusion	This study
pNZchimer6	Cm^r; P*spaS*$_{mut}$-S_{TMS123}-Y_{TMS45} fusion	This study
pNZchimer1his$_6$	Like pNZ8901, contains the Y_{TMS1}-$S_{TMS2345}$ fusion with a His_6 tag at the 3′ end	This study
pNZchimer7	Cm^r; P*spaS*$_{mut}$-Y_{P1}-$S_{TMS12345}$ fusion	This study
pNZchimer8	Cm^r; P*spaS*$_{mut}$-S_{P1}-$Y_{TMS12345}$ fusion	This study
Strains		
E. coli DH5α	*supE44 hsdR14 recA1 endA1 gyrA96 thi-1 relA1* Δ*lacU169* (ϕ80*lacZ*ΔM15); K-12 derivative	Laboratory stock
B. subtilis		
168	*trpC2*	23
ATCC 6633	Subtilin producer	24
NZ8900	168 *spaRK*; Kan^r	14
MS001	168 *spoIIIJ*::Tet^r	9
MS005	MS001 *yqjG*::Spc^r *lacA*::P_{xylA}-*yqjG*; Em^r	9
YG000	MS005 *amyE*::*spaRK*; Kan^r	This study
YG001	MS001 *amyE*::$P_{hyper\text{-}spank}$-Y_{P1}-$S_{TMS2345}$; Spc^r	This study
YG002	MS001 *amyE*::$P_{hyper\text{-}spank}$-Y_{TMS1}-$S_{TMS2345}$; Spc^r	This study
YG003	MS001 *amyE*::$P_{hyper\text{-}spank}$-Y_{TMS12}-S_{TMS345}; Spc^r	This study
YG004	MS001 *amyE*::$P_{hyper\text{-}spank}$-Y_{TMS123}-S_{TMS45}; Spc^r	This study
YG005	MS001 *amyE*::$P_{hyper\text{-}spank}$-S_{P1}-$Y_{TMS12345}$; Spc^r	This study
YG006	MS001 *amyE*::$P_{hyper\text{-}spank}$-S_{TMS1}-$Y_{TMS2345}$; Spc^r	This study
YG007	MS001 *amyE*::$P_{hyper\text{-}spank}$-S_{TMS12}-Y_{TMS345}; Spc^r	This study
YG008	MS001 *amyE*::$P_{hyper\text{-}spank}$-S_{TMS123}-Y_{TMS45}; Spc^r	This study
YG009	MS001 *amyE*::$P_{hyper\text{-}spank}$-$S_{TMS1345}$-Y_{TMS2}; Spc^r	This study
YG010	MS001 *amyE*::$P_{hyper\text{-}spank}$-$Y_{TMS1345}$-S_{TMS2}; Spc^r	This study
YG011	MS001 *amyE*::$P_{hyper\text{-}spank}$-*spoIIIJ*; Spc^r	This study
YG012	MS001 *amyE*::$P_{hyper\text{-}spank}$-*yqjG*; Spc^r	This study
YG013	MS001 *amyE*::$P_{hyper\text{-}spank}$-$S_{TMS1345}$-$Y_{TMS2syn}$; Spc^r	This study

To introduce the chimeric genes into the *amyE* locus, the pDR plasmids were transformed into *B. subtilis* MS001(Δ*spoIIIJ*) (9) with selection for spectinomycin. Successful integration at the *amyE* locus was verified by an inability to metabolize starch.

To introduce the *spaRK* genes into the *amyE* locus of *B. subtilis* MS005 (Δ*yqjG* Δ*spoIIIJ* P_{xylA}-*yqjG*) (9), DNA of NZ8900 harboring these genes (14) was transformed into MS005. Kanamycin-resistant colonies were analyzed for the loss of the ability to metabolize starch to verify integration at the *amyE* locus, and the resulting strain was designated YG000. For overexpression of the chimeric genes in *B. subtilis* YG000 (MS005 *amyE*::*spaRK*), the SURE expression system was used (14). Primer pairs chimera123fw/chimera123rev and chimera456fw/chimera456rev were

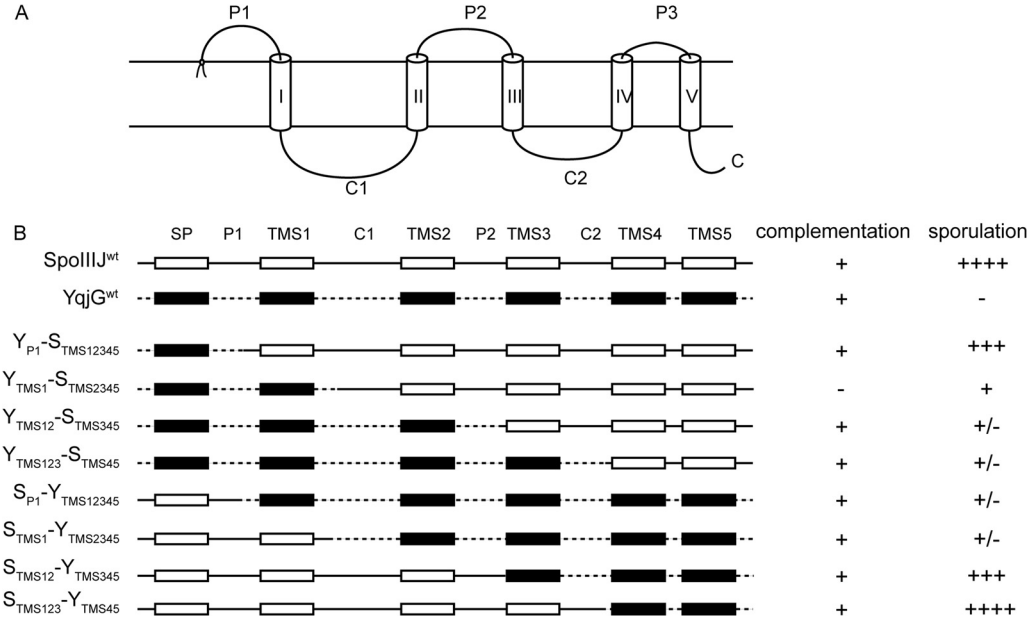


FIG 1 SpoIIIJ-YqjG/YqjG-SpoIIIJ chimeras can functionally restore the growth defect of *B. subtilis* YG000 (Δ*yqjG* Δ*spoIIIJ* P_{xylA}-*yqjG amyE*::*spaRK*). (A) Schematic representation of the SpoIIIJ/YqjG topology. (B) Construction of the chimeric fusions. TMS, transmembrane segment. $SpoIIIJ^{wt}$, wild-type SpoIIIJ; $YqjG^{wt}$, wild-type YqjG; S, wild-type SpoIIIJ; Y, wild-type YqjG. SpoIIIJ TMSs are represented as white boxes, YqjG TMSs are shown as black boxes, SpoIIIJ loop segments are shown as lines, and YqjG loops are shown as dashed lines. +++, viable spores present in a 10^{-3} dilution; ++, viable spores present in a 10^{-2} dilution; +, viable spores present in a 10^{0} dilution; +/−, viable spores occasionally present in 10^{0} dilutions, depending on the experiment; −, no viable spores detected. The experiment was performed at least 4 times for each strain, hence the +/− result.

used for amplification of the chimeric fragments from the plasmids pDRchimer1 to pDRchimer8. After cleavage with NcoI and XbaI, the PCR products were ligated into the corresponding sites of pNZ8901, yielding the corresponding pNZchimer* plasmids (Table 1), and transformed into *B. subtilis* YG000. Control constructs containing full-length *spoIIIJ* and *yqjG* were constructed in the same way and transformed into YG000. The inducer subtilin, which was extracted from a fresh culture supernatant of *B. subtilis* ATCC 6633 (14), was added to induce overexpression.

To add a six-histidine tag to the 3′ end of the chimeric gene with TMS1 of YqjG and TMS2 to TMS5 of SpoIIIJ, the chimeric fragment with NheI and SphI sites was amplified by using the primer set chimers123fw/chimers123hisrev (see Table S1 in the supplemental material). After double digestion and ligation, the resulting plasmid pNZchimer1his was obtained. All plasmids used in this study were verified by sequencing.

Growth complementation. *B. subtilis* strain YG000 (MS005 *amyE*::*spaRK*) was transformed with the pNZ8901-derived plasmids carrying the YqjG/SpoIIIJ chimeras. Transformants were selected on LB plates supplemented with chloramphenicol (10 μg/ml) and 0.2% (wt/vol) xylose and restreaked onto LB plates supplemented with spectinomycin (100 μg/ml), erythromycin (2 μg/ml), tetracycline (6 μg/ml), and either 1% (vol/vol) subtilin or 0.5% (wt/vol) glucose. Plasmids that allowed growth of YG000 on plates containing subtilin, but not on plates containing glucose, were regarded as complementation positive.

Protein expression and immunoblotting. In order to test the expression of the chimeric proteins in *B. subtilis*, a culture grown overnight was diluted 1:50 into fresh LB medium supplemented with chloramphenicol (10 μg/ml) and grown to an optical density at 600 nm (OD_{600}) of 1.0. Protein expression was induced by the addition of 0.5% (vol/vol) subtilin, and cell growth was continued for 2 h. Cells were harvested, and the membrane fraction was isolated as described previously (4). The protein content of the membrane fraction was analyzed on 15% SDS-PAGE gels, followed by Western blotting and detection of His-tagged proteins using an anti-His antibody (Sigma-Aldrich), according to standard protocols.

Sporulation efficiency. To measure the efficiency of sporulation, a fresh culture of *B. subtilis* grown overnight was diluted into 15 ml prewarmed growth medium (15) to an OD_{600} of 0.1. Expression of the chimeric proteins was induced by the addition of 50 μM isopropyl-β-D-1-thiogalactopyranoside (IPTG) to the culture, and cells were grown to an OD_{600} of ~0.7 to 0.8 at 37°C under vigorous shaking. Cells were collected by centrifugation (3,000 × *g* for 15 min at 37°C) and resuspended in 15 ml prewarmed sporulation medium (15), and incubation at 37°C was continued for an additional 24 h. The culture was split, with one part being heated at 80°C for 20 min. Subsequently, 10-fold dilution series of the heated and nonheated cell suspensions were made, and 2 μl of each dilution was spotted onto LB plates.

RESULTS

Complementation of the growth defect of a *spoIIIJ yqjG* double deletion strain by chimeric proteins. SpoIIIJ and YqjG both contain the conserved hydrophobic core consisting of five transmembrane segments (TMSs) of the YidC/OxaI/Alb3 protein family, preceded by a putative lipoprotein signal peptide (Fig. 1A). To determine whether specific domains of SpoIIIJ determine its essential role in sporulation, a set of SpoIIIJ-YqjG and YqjG-SpoIIIJ chimeric proteins was constructed by replacing transmembrane segments of SpoIIIJ with the corresponding parts of YqjG and vice versa (Fig. 1B). The overlapping PCR technique used to swap TMSs also resulted in partial replacement of flanking regions (Fig. 1B; see also Fig. S1A in the supplemental material). The functionality of the chimeras was tested by determining their capacity to complement the growth defect of a *spoIIIJ yqjG* double deletion *B. subtilis* strain. The chimeric genes were cloned into plasmid pNZ8901, which allows for subtilin-inducible expression, and transformed into *B. subtilis* YG000 (Δ*yqjG* Δ*spoIIIJ* P_{xylA}-*yqjG amyE*::*spaRK*). In YG000, the chromosomal copies of *yqjG* and *spoIIIJ* are deleted, while a xylose-inducible *yqjG* gene is integrated into the genomic *lacA* locus. Either SpoIIIJ (abbreviated S) or YqjG (abbreviated Y) is sufficient for cell viability, while a double

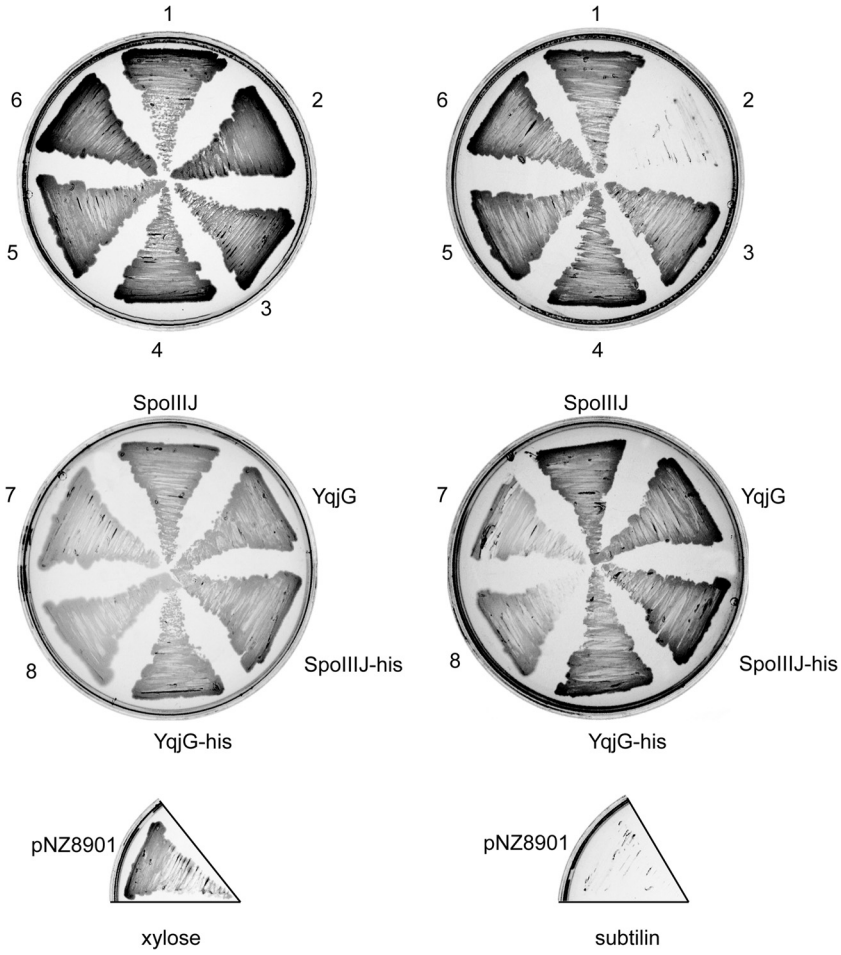


FIG 2 Complementation of YG000 with plasmid-encoded SpoIIIJ-YqjG/YqjG-SpoIIIJ chimeras. Cells containing Y_{P1}-$S_{TMS12345}$ (section 1), Y_{TMS1}-$S_{TMS2345}$ (section 2), Y_{TMS12}-S_{TMS345} (section 3), Y_{TMS123}-S_{TMS45} (section 4), S_{P1}-$Y_{TMS12345}$ (section 5), S_{TMS1}-$Y_{TMS2345}$ (section 6), S_{TMS12}-Y_{TMS345} (section 7), and S_{TMS123}-Y_{TMS45} (section 8) were streaked onto LB plates supplemented with the corresponding antibiotics and 0.2% (wt/vol) xylose or 1% (vol/vol) subtilin. The empty plasmid (pNZ8901) and plasmids encoding SpoIIIJ, YqjG, SpoIIIJ-His, and YqjG-His are shown as controls.

deletion is lethal (11). Therefore, growth of YG000 is strictly xylose dependent. Empty plasmid pNZ8901 and plasmids containing the full-length *spoIIIJ* and *yqjG* genes were transformed into YG000 to serve as negative and positive controls, respectively. Expression of *spoIIIJ*, *yqjG*, and the chimeras was induced by the addition of subtilin to the growth medium. As expected, the negative control (pNZ8901) grew only on LB plates supplemented with xylose (Fig. 2), while cells carrying pNZspoIIIJ or pNZyqjG were viable in the presence of either xylose or subtilin. Notably, except for the Y_{TMS1}-$S_{TMS2345}$ mutant, all the chimeras supported growth of the *spoIIIJ yqjG* double deletion strain, which demonstrated that these chimeras can substitute for the function of SpoIIIJ/YqjG during vegetative growth of *B. subtilis*.

The observation that Y_{TMS1}-$S_{TMS2345}$ could not complement the *spoIIIJ yqjG* double deletion strain suggests that the chimera is either not functional or not expressed. To discriminate between these possibilities, a C-terminally His-tagged Y_{TMS1}-$S_{TMS2345}$ chimeric fusion was created and transformed into YG000. Subtilin-induced expression of Y_{TMS1}-$S_{TMS2345}$-His also did not restore the growth defect of YG000, but the protein was expressed, as shown by Western blotting (see Fig. S2 in the supplemental material). Apparently, this fusion protein is inactive. Taken together, these results demonstrate that all but one of the chimeras can functionally replace SpoIIIJ/YqjG during vegetative growth.

Rescue of the sporulation defect of a *B. subtilis spoIIIJ* strain by chimeras. SpoIIIJ is essential for sporulation, and YqjG cannot rescue the sporulation defect of a *spoIIIJ* deletion strain. To analyze whether the chimeric fusion proteins were supporting sporulation, the genes were placed under the control of the IPTG-inducible $P_{hyper\text{-}spank}$ promoter and integrated into the *amyE* locus of *B. subtilis* MS001 (Δ*spoIIIJ*). Cells were grown to the exponential phase, transferred into sporulation medium, and allowed to sporulate for 24 h. Cultures were subjected to a heat shock at 80°C for 20 min, and the sporulation efficiency was assayed by the ability to grow on LB medium after serial dilution. As shown in Fig. 3, cells that were not subjected to heat shock grew robustly on the LB plates and exhibited a similar growth pattern. However, after heat shock, no growth was observed for *B. subtilis* MS001 (Δ*spoIIIJ*). An IPTG-inducible copy of *spoIIIJ* at the genomic *amyE* locus could functionally complement the *spoIIIJ* deletion defect, whereas a similar construct with *yqjG* could not (Fig. 3A). Replacement of the putative signal peptide and periplasmic loop P1 of SpoIIIJ with those of YqjG did not affect the sporulation efficiency (Y_{P1}-$S_{TMS12345}$) (Fig. 3B), indicating that this region is not

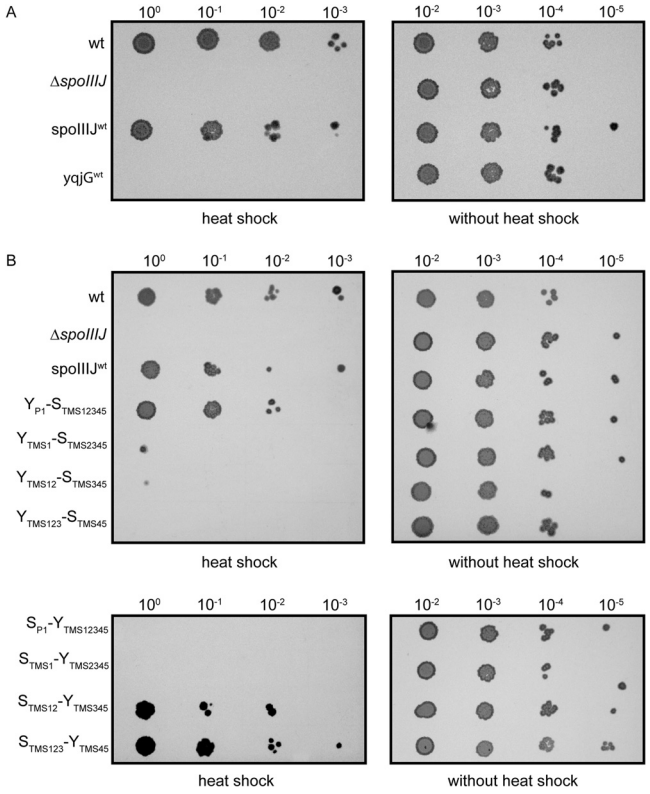


FIG 3 SpoIIIJ-YqjG/YqjG-SpoIIIJ rescues a *spoIIIJ* sporulation phenotype to different extents. A series of SpoIIIJ-YqjG/YqjG-SpoIIIJ chimeric fusions were integrated into the genomic *amyE* locus of MS001 (Δ*spoIIIJ*), and cells were allowed to sporulate. Cell suspensions were adjusted to the same OD_{600} and subjected to heat shock at 80°C for 20 min or not, after which a 10-fold dilution series was spotted onto LB plates. wt, wild-type *B. subtilis* 168; Δ*spoIIIJ*, MS001 (Δ*spoIIIJ*); $SpoIIIJ^{wt}$, MS001 *amyE*::P_{HS}-*spoIIIJ*; $YqjG^{wt}$, MS001 *amyE*::P_{HS}-*yqjG*. Dilution factors are indicated above the panels.

required for the sporulation-specific function of SpoIIIJ. However, when the region including TMS1 and -2 of SpoIIIJ was replaced with the corresponding part of YqjG (Y_{TMS12}-S_{TMS345}) (Fig. 1B), the sporulation efficiency was dramatically reduced (Y_{TMS12}-S_{TMS345}) (Fig. 3B [please note that Y_{TMS1}-$S_{TMS2345}$ was not functional in the complementation assay]). This suggests that TMS1 and -2 of SpoIIIJ are involved in the sporulation-specific function of SpoIIIJ.

Next, we tested whether N-terminal fragments of SpoIIIJ could enable YqjG to function in sporulation. A chimera in which the putative signal peptide and P1 domain of YqjG were replaced with the corresponding regions of SpoIIIJ (S_{P1}-$Y_{TMS12345}$) did not support sporulation. Likewise, extending the replacement to TMS1 (S_{TMS1}-$Y_{TMS2345}$) did not enable sporulation. The sporulation efficiency increased dramatically when both TMS1 and TMS2 of YqjG were replaced with the corresponding fragment of SpoIIIJ (S_{TMS12}-Y_{TMS345}), again suggesting that the region comprising TMS1 and -2 of SpoIIIJ plays an important role in sporulation specificity. When the replacement was extended to TMS3 (S_{TMS123}-Y_{TMS45}), the sporulation efficiency increased even further to a level that was almost similar to that of wild-type SpoIIIJ. A summary of the sporulation efficiency results from the tested chimeras is shown in Fig. 1B. Taken together, the data suggest that the putative signal peptide and periplasmic loop 1 are not critical for the role of SpoIIIJ in sporulation, while the region comprising TMS1 and TMS2 seems to play a key role. TMS3 may function cooperatively with TMS1 and TMS2, since the YqjG mutant (S_{TMS123}-Y_{TMS45}) yielded exactly the same sporulation efficiency as that of wild-type SpoIIIJ.

The domain comprising TMS2 of SpoIIIJ plays a crucial role in sporulation specificity. To analyze whether TMS2 of SpoIIIJ is the sole determinant for the sporulation-specific role of SpoIIIJ, TMS2 and part of its flanking loops were swapped between SpoIIIJ and YqjG ($S_{TMS1345}$-Y_{TMS2} and $Y_{TMS1345}$-S_{TMS2}) (Fig. 4A). When cloned into the subtilin-inducible expression vector pNZ8901, both mutants were able to complement the growth defect of double deletion strain YG000 (Fig. 4B). As shown in Fig. 4C, replacement of the fragment comprising TMS2 and its flanking loops ($S_{TMS1345}$-Y_{TMS2}) blocked the sporulation activity of SpoIIIJ, confirming that this is an essential region that cannot be functionally replaced by that of YqjG. However, replacing this domain of YqjG with that of SpoIIIJ was not sufficient to enable YqjG to support sporulation ($Y_{TMS1345}$-S_{TMS2}) (Fig. 4C). Our overlapping PCR technique replaced not only TMS2 but also parts of the TMS2-flanking regions. To narrow down the function of TMS2, a synthetic construct comprising SpoIIIJ with solely TMS2 of YqjG was generated. This protein was functional in complementation of the growth defect of YG000 (Fig. 4B) but was also affected in sporulation efficiency ($S_{TMS1345}$-$Y_{TMS2syn}$) (Fig. 4C) although to a lesser extent than the construct containing SpoIIIJ TMS2 in combination with the flanking loops. Taken together, these results suggest that the domain comprising TMS2 and its hydrophilic flanking loops constitutes an essential, but not the only, determinant for the sporulation-specific activity of SpoIIIJ.

DISCUSSION

The members of the YidC/OxaI/Alb3 protein family have a dedicated role in the biogenesis of membrane proteins and are functionally exchangeable (1–3). The *B. subtilis* YidC homologues SpoIIIJ and YqjG share a conserved and overlapping function in membrane protein insertion and assembly (4). Besides their overlapping functions during vegetative growth, SpoIIIJ plays a specific role in sporulation (7). Here we examined which regions of SpoIIIJ are critical for the sporulation specificity of SpoIIIJ by systematically replacing the TMSs of SpoIIIJ with those of YqjG and vice versa. Our data suggest an essential role of TMS2 and its flanking loops in sporulation specificity, while the two C-terminal TMSs do not seem to contribute to the SpoIIIJ-specific sporulation function. Remarkably, replacement of TMS1 of SpoIIIJ with that of YqjG inactivates SpoIIIJ in complementing the growth defect of the *spoIIIJ yqjG* double deletion strain, whereas it retained a slight activity in the sporulation assay. Since the chimera is indeed expressed in the cell, we speculate that the replacement of TMS1 causes a conformational change in SpoIIIJ that inactivates the protein to the extent that it can no longer support cell viability. YqjG fulfills a specific function in competence development; however, this function is not as critical as the role of SpoIIIJ in sporulation, as even in the absence of YqjG, cells still become competent (9).

The expression of *spoIIIJ* is essential for the activation of the forespore-specific factor σ^G, and in the absence of SpoIIIJ, sporulation is arrested soon after the completion of the engulfment process (7). The activation of σ^G also requires the expression of the *spoIIIAA–spoIIIAH* operon, encoding eight proteins, seven of

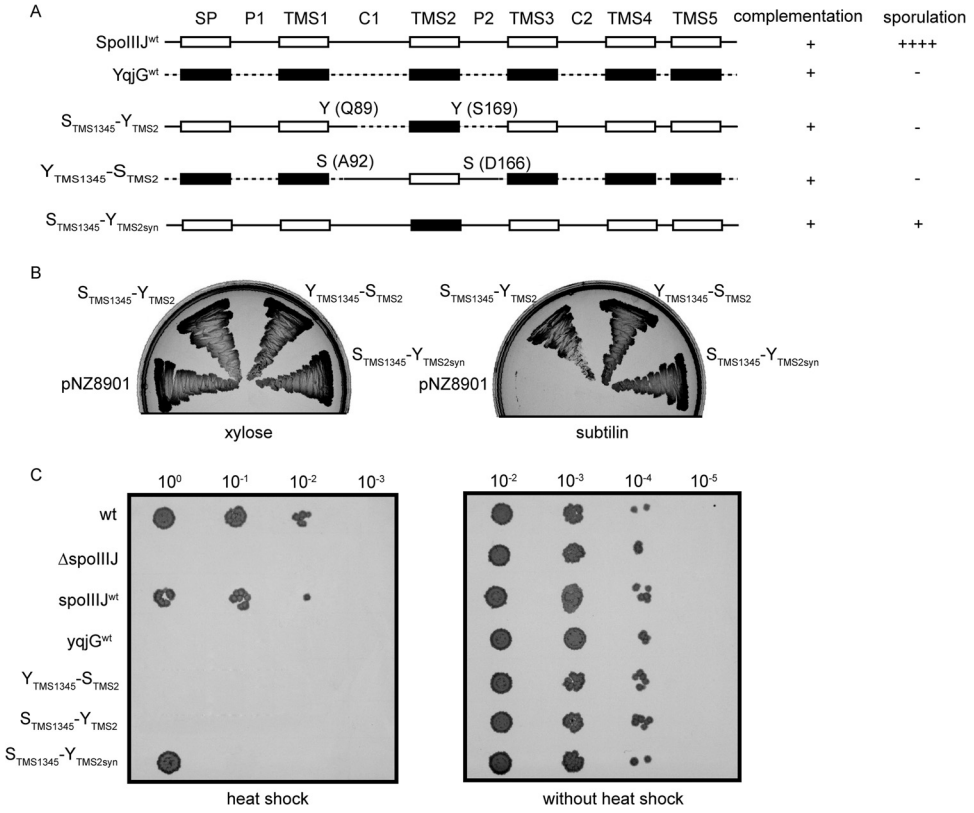


FIG 4 TMS2 of SpoIIIJ is not sufficient to convert YqjG into a sporulation-specific insertase. (A) Construction of TMS2-replaced SpoIIIJ and YqjG mutants. (B) Growth complementation of YG000 (Δ*yqjG* Δ*spoIIIJ* P_{xylA}-*yqjG amyE*::*spaRK*) with plasmids encoding $S_{TMS1345}$-Y_{TMS2}, $Y_{TMS1345}$-S_{TMS2}, and $S_{TMS1345}$-$Y_{TMS2syn}$. Empty plasmid pNZ8901 served as a negative control. (C) Sporulation ability test of TMS2-replaced SpoIIIJ and YqjG mutants. wt, wild-type *B. subtilis* 168; Δ*spoIIIJ*, MS001 (Δ*spoIIIJ*); $SpoIIIJ^{wt}$, MS001 *amyE*::P_{HS}-*spoIIIJ*; $YqjG^{wt}$, MS001 *amyE*::P_{HS}-*yqjG*. Dilution factors are indicated above the panels. ++++, viable spores present in a 10^{-3} dilution; +, viable spores present in a 10^{0} dilution; −, no viable spores detected. The experiment was performed at least 4 times for each strain.

which are membrane targeted (16, 17). SpoIIIJ may interact directly with the membrane protein SpoIIIAE and facilitate its correct folding or assembly following the insertion step (10). Another *spoIIIA*-encoded membrane product, SpoIIIAH, is also proposed to play a key role in σ^G activation (18) and may thus be a potential substrate for SpoIIIJ. SpoIIIAH, which carries a single transmembrane segment, interacts with the forespore-expressed SpoIIQ protein at the sporulation septum to form a SpoIIIAH/SpoIIQ channel, which is supposed to export the anti-sigma factor proteins of the forespore, thereby activating σ^G (17, 19). A previous proteomics study of the *spoIIIJ* mutant revealed an increased expression level of SpoIIIAH, implying that SpoIIIJ might function in the correct folding of SpoIIIAH rather than its insertion into the membrane (9). However, further investigations are needed to verify this hypothesis. Sporulation is a highly sophisticated development process involving compartment-specific gene activation and intercellular communication between the mother and forespore cells, so as-yet-unknown products involved in sporulation may represent potential substrates for SpoIIIJ.

TMS2 of the conserved hydrophobic core of the YidC/OxaI/Alb3 family is the most conserved part of the protein. Mutations in TMS3 of *E. coli* YidC (which corresponds to TMS2 in SpoIIIJ) cause a cold-sensitive phenotype (20), while cross-linking results suggest that YidC TMS3 contacts nascent inner membrane proteins, possibly providing a generic docking site for membrane proteins to insert into the membrane (21). Here we found that the corresponding TMS2 of SpoIIIJ and its flanking regions are critical for sporulation specificity, cooperating with TMS1 and TMS3 to achieve full functionality. The specificity seems to result from both the TMS and its surrounding hydrophilic loops, since replacement of TMS2 alone reduces, but does not completely block, sporulation activity. This is in accordance with previous findings which showed that YidC is very resilient to amino acid substitutions and even tolerates the exchange of two TMSs (equivalent to SpoIIIJ TMS3 and -4) for totally unrelated TMSs (22). Comparison of TMS2 of YqjG and that of SpoIIIJ shows that they are highly conserved, while the observed substitutions only marginally affected the overall hydrophobicity, except for methionine 141 of SpoIIIJ, which was substituted for serine 149 in YqjG. However, the M141S mutation in SpoIIIJ did not inactivate its sporulation-specific function, nor did the S149M mutation in YqjG result in a gain of this function (see Fig. S1C in the supplemental material). Our data suggest that the area comprising SpoIIIJ TMS1, -2, and -3 and the connecting loops is present as a core fold that is critical for functioning and specificity. Further studies are required to pinpoint what makes SpoIIIJ sporulation specific and fundamentally different from YqjG and, for example, YidC. Identification of a true SpoIIIJ substrate and development of an *in vitro* insertion assay for this substrate are necessary tools for these studies.

ACKNOWLEDGMENTS

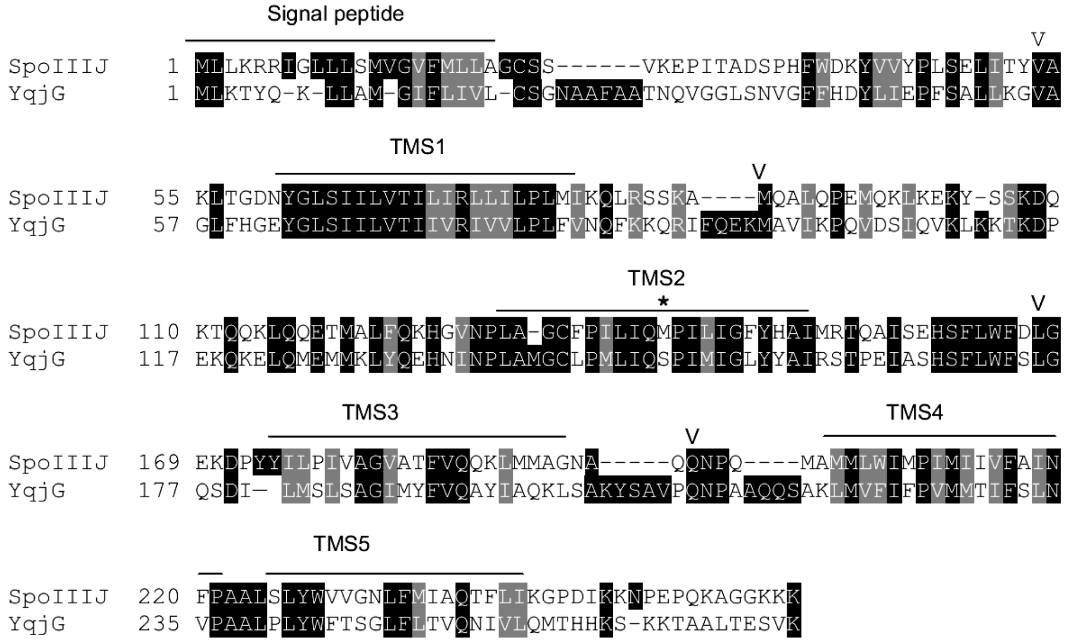
We thank David Rudner (Harvard Medical School, USA) for the kind gift of plasmid pDR111.

This work was funded by a Chinese scholarship (CSC) grant to Y.P. and by a Vidi grant from the Netherlands Organization for Scientific Research to D.-J.S.

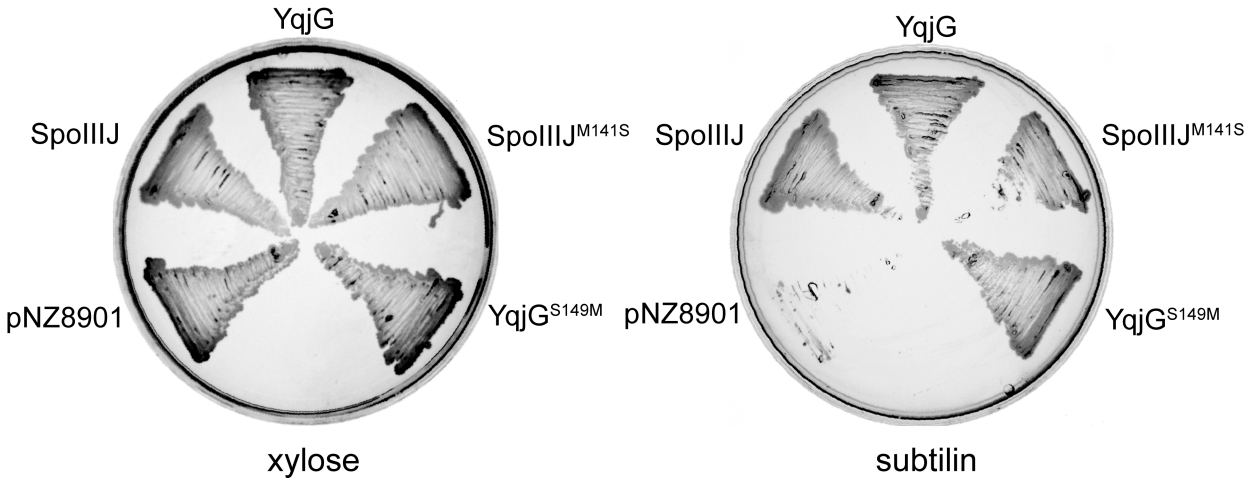
REFERENCES

1. **Wang P, Dalbey RE.** 2011. Inserting membrane proteins: the YidC/OxaI/Alb3 machinery in bacteria, mitochondria, and chloroplasts. Biochim. Biophys. Acta **1808:**866–875. http://dx.doi.org/10.1016/j.bbamem.2010.08.014.
2. **Funes S, Kauff F, van der Sluis EO, Ott M, Herrmann JM.** 2011. Evolution of YidC/OxaI/Alb3 insertases: three independent gene duplications followed by functional specialization in bacteria, mitochondria and chloroplasts. Biol. Chem. **392:**13–19. http://dx.doi.org/10.1515/BC.2011.013.
3. **Van Der Laan M, Nouwen NP, Driessen AJM.** 2005. YidC—an evolutionary conserved device for the assembly of energy-transducing membrane protein complexes. Curr. Opin. Microbiol. **8:**182–187. http://dx.doi.org/10.1016/j.mib.2005.02.004.
4. **Saller MJ, Fusetti F, Driessen AJM.** 2009. *Bacillus subtilis* SpoIIIJ and YqjG function in membrane protein biogenesis. J. Bacteriol. **191:**6749–6757. http://dx.doi.org/10.1128/JB.00853-09.
5. **Dalbey RE, Kuhn A.** 2004. YidC family members are involved in the membrane insertion, lateral integration, folding, and assembly of membrane proteins. J. Cell Biol. **166:**769–774. http://dx.doi.org/10.1083/jcb.200405161.
6. **Tjalsma H, Bron S, van Dijl JM.** 2003. Complementary impact of paralogous OxaI-like proteins of *Bacillus subtilis* on post-translocational stages in protein secretion. J. Biol. Chem. **278:**15622–15632. http://dx.doi.org/10.1074/jbc.M301205200.
7. **Errington J, Appleby L, Daniel RA, Goodfellow H, Partridge SR, Yudkin MD.** 1992. Structure and function of the *spoIIIJ* gene of *Bacillus subtilis*: a vegetatively expressed gene that is essential for sigma G activity at an intermediate stage of sporulation. J. Gen. Microbiol. **138:**2609–2618. http://dx.doi.org/10.1099/00221287-138-12-2609.
8. **Chiba S, Lamsa A, Pogliano K.** 2009. A ribosome-nascent chain sensor of membrane protein biogenesis in *Bacillus subtilis*. EMBO J. **28:**3461–3475. http://dx.doi.org/10.1038/emboj.2009.280.
9. **Saller MJ, Otto A, Berrelkamp-Lahpor GA, Becher D, Hecker M, Driessen AJM.** 2011. *Bacillus subtilis* YqjG is required for genetic competence development. Proteomics **11:**270–282. http://dx.doi.org/10.1002/pmic.201000435.
10. **Serrano M, Côrte L, Opdyke J, Charles P, Henriques AO.** 2003. Expression of *spoIIIJ* in the prespore is sufficient for activation of σ^G and for sporulation in *Bacillus subtilis*. J. Bacteriol. **185:**3905–3917. http://dx.doi.org/10.1128/JB.185.13.3905-3917.2003.
11. **Murakami T, Haga K, Takeuchi M, Sato T.** 2002. Analysis of the *Bacillus subtilis spoIIIJ* gene and its paralogue gene, *yqjG*. J. Bacteriol. **184:**1998–2004. http://dx.doi.org/10.1128/JB.184.7.1998-2004.2002.
12. **Serrano M, Vieira F, Moran CP, Henriques AO.** 2008. Processing of a membrane protein required for cell-to-cell signaling during endospore formation in *Bacillus subtilis*. J. Bacteriol. **190:**7786–7796. http://dx.doi.org/10.1128/JB.00715-08.
13. **Jenkinson HF.** 1983. Altered arrangement of proteins in the spore coat of a germination mutant of *Bacillus subtilis*. J. Gen. Microbiol. **129:**1945–1958.
14. **Bongers R.** 2005. Development and characterization of a subtilin-regulated expression system in *Bacillus subtilis*: strict control of gene expression by addition of subtilin. Appl. Environ. Microbiol. **71:**8818–8824. http://dx.doi.org/10.1128/AEM.71.12.8818-8824.2005.
15. **Scheffers D-J.** 2005. Dynamic localization of penicillin-binding proteins during spore development in *Bacillus subtilis*. Microbiology **151:**999–1012. http://dx.doi.org/10.1099/mic.0.27692-0.
16. **Kellner EM, Decatur A, Moran CP.** 1996. Two-stage regulation of an anti-sigma factor determines developmental fate during bacterial endospore formation. Mol. Microbiol. **21:**913–924. http://dx.doi.org/10.1046/j.1365-2958.1996.461408.x.
17. **Higgins D, Dworkin J.** 2012. Recent progress in *Bacillus subtilis* sporulation. FEMS Microbiol. Rev. **36:**131–148. http://dx.doi.org/10.1111/j.1574-6976.2011.00310.x.
18. **Guillot C, Moran CP.** 2007. Essential internal promoter in the *spoIIIA* locus of *Bacillus subtilis*. J. Bacteriol. **189:**7181–7189. http://dx.doi.org/10.1128/JB.00915-07.
19. **Blaylock B, Jiang X, Rubio A, Moran CP, Pogliano K.** 2004. Zipper-like interaction between proteins in adjacent daughter cells mediates protein localization. Genes Dev. **18:**2916–2928. http://dx.doi.org/10.1101/gad.1252704.
20. **Yuan J, Phillips GJ, Dalbey RE.** 2007. Isolation of cold-sensitive *yidC* mutants provides insights into the substrate profile of the YidC insertase and the importance of transmembrane 3 in YidC function. J. Bacteriol. **189:**8961–8972. http://dx.doi.org/10.1128/JB.01365-07.
21. **Yu Z, Koningstein G, Pop A, Luirink J.** 2008. The conserved third transmembrane segment of YidC contacts nascent *Escherichia coli* inner membrane proteins. J. Biol. Chem. **283:**34635–34642. http://dx.doi.org/10.1074/jbc.M804344200.
22. **Jiang F, Chen M, Yi L, de Gier J-W, Kuhn A, Dalbey RE.** 2003. Defining the regions of *Escherichia coli* YidC that contribute to activity. J. Biol. Chem. **278:**48965–48972. http://dx.doi.org/10.1074/jbc.M307362200.
23. **Kunst F, Ogasawara N, Moszer I, Albertini A, Alloni MG, Azevedo V, Bertero MG, Bessières P, Bolotin A, Borchert S, Borriss R, Boursier L, Brans A, Braun M, Brignell SC, Bron S, Brouillet S, Bruschi CV, Caldwell B, Capuano V, Carter NM, Choi SK, Codani JJ, Connerton IF, Danchin A.** 1997. The complete genome sequence of the gram-positive bacterium *Bacillus subtilis*. Nature **390:**249–256. http://dx.doi.org/10.1038/36786.
24. **Duitman EH, Hamoen LW, Rembold M, Venema G, Seitz H, Saenger W, Bernhard F, Reinhardt R, Schmidt M, Ullrich C, Stein T, Leenders F, Vater J.** 1999. The mycosubtilin synthetase of *Bacillus subtilis* ATCC6633: a multifunctional hybrid between a peptide synthetase, an amino transferase, and a fatty acid synthase. Proc. Natl. Acad. Sci. U. S. A. **96:**13294–13299. http://dx.doi.org/10.1073/pnas.96.23.13294.

A


Signal peptide
V
SpoIIIJ 1 MLLKRRIGLLLSMVGVFMLLAGCSS------VKEPITADSPHFWDKYVVYPLSELITYVA
YqjG 1 MLKTYQ-K-LLAM-GIFLIVL-CSGNAAFAATNQVGGLSNVGFFHDYLIEPFSALLKGVA
TMS1
V
SpoIIIJ 55 KLTGDNYGLSIILVTILIRLLILPLMIKQLRSSKA----MQALQPEMQKLKEKY-SSKDQ
YqjG 57 GLFHGEYGLSIILVTIIVRIVVLPLFVNQFKKQRIFQEKMAVIKPQVDSIQVKLKKTKDP
TMS2
*
V
SpoIIIJ 110 KTQQKLQQETMALFQKHGVNPLA-GCFPILIQMPILIGFYHAIMRTQAISEHSFLWFDLG
YqjG 117 EKQKELQMEMMKLYQEHNINPLAMGCLPMLIQSPIMIGLYYAIRSTPEIASHSFLWFSLG
TMS3
V
TMS4
SpoIIIJ 169 EKDPYYIIPIVAGVATFVQQKLMMAGNA-----QQNPQ----MAMMLWIMPIMIIVFAIN
YqjG 177 QSDI- LMSLSAGIMYFVQAYIAQKLSAKYSAVPQNPAAQQSAKLMVFIFPVMMTIFSLN
TMS5
SpoIIIJ 220 FPAALSLYWVVGNLFMIAQTFLIKGPDIKKNPEPQKAGGKKK
YqjG 235 VPAALPLYWFTSGLFLTVQNIVLQMTHHKS-KKTAALTESVK

B


YqjG
SpoIIIJ
SpoIIIJ^M141S
pNZ8901
YqjG^S149M
xylose
YqjG
SpoIIIJ
SpoIIIJ^M141S
pNZ8901
YqjG^S149M
subtilin

C

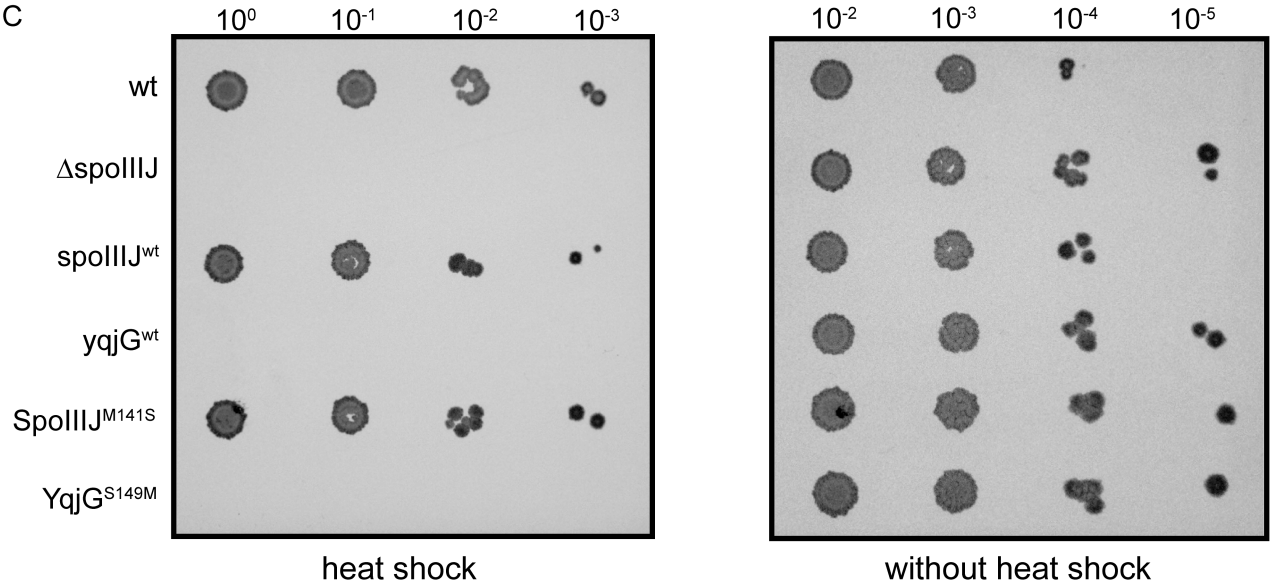

10^0
10^-1
10^-2
10^-3
wt
ΔspoIIIJ
spoIIIJ^wt
yqjG^wt
SpoIIIJ^M141S
YqjG^S149M
heat shock
10^-2
10^-3
10^-4
10^-5
without heat shock

Figure S1. Single amino acid mutagenesis in the TMS2 of the SpoIIIJ has no effect on sporulation ability. A) Alignment of the *B. sutbilis* SpoIIIJ and YqjG. The alignment is generated using T-Coffee and Boxshade. Putative transmembrane segments (TMS) are indicated with straight lines. The residue in TMS2 chosen for mutation is indicated with * and the amino acid sites chosen for chimeras construction are indicated with V. B) Growth complementation of YG000 (Δ*yqjG*, Δ*spoIIIJ*, P_{xylA}*yqjG, amyE::spaRK*) with plasmids encoding $SpoIIIJ^{M141S}$ and $YqjG^{S149M}$. Empty plasmid pNZ8901 and plasmids containing SpoIIIJ and YqjG served as controls. C) Sporulation ability test of the single amino acid mutated SpoIIIJ. wt, wild type *B. subtilis* 168; Δ*spoIIIJ*, MS001 (Δ*spoIIIJ*); $SpoIIIJ^{wt}$, MS001, *amyE*::P_{HS}-*spoIIIJ*; $YqjG^{wt}$, MS001, *amyE*::P_{HS}-*yqjG*. Dilution factors are indicated above the panels.

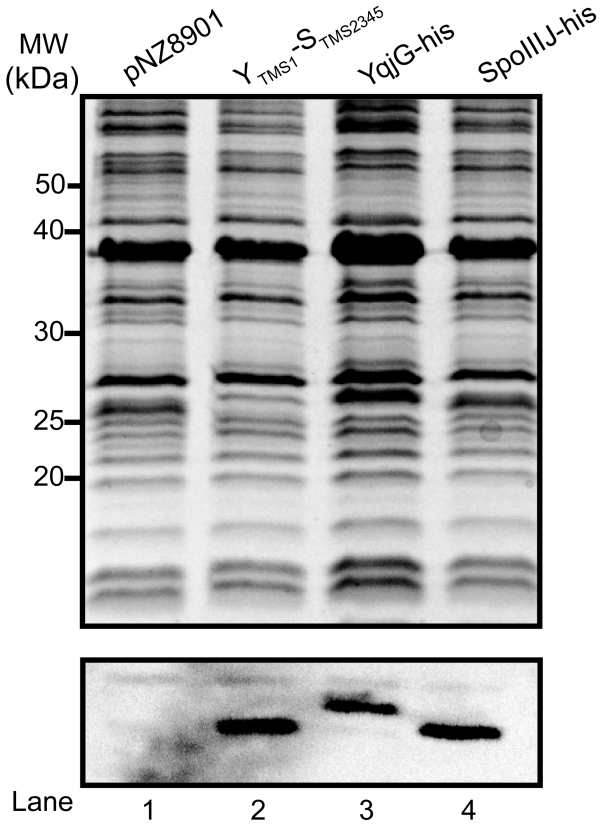


Figure S2. Chimeric protein Y_{TMS1}-$S_{TMS2345}$ was expressed in YG000 (Δ*yqjG*, Δ*spoIIIJ*, P_{xylA}*yqjG, amyE::spaRK*) . Cultures harboring plasmids pNZ8901 (lane1, empty control), pNZchimer1his (lane 2, *Y_{TMS1}-$S_{TMS2345}$his*), pNZyqjGhis and pNZspoIIIJhis (lanes 3 and 4, positive controls) were grown as described in the Materials and methods, and expression of the chimeric protein Y_{TMS1}-$S_{TMS2345}$ was monitored by SDS-PAGE and immunoblotting using anti-His antibody.

TABLE S1. List of primers

Primer name	Nucleotide Sequence (5'-3')
YqjGfw	GCGGCCAAGCTTAGGAGGAGAACAAAATTG
YqjGrev	GCCTGCGCTAGCTTATTTCACCGACTCAGTAAGAG
Spofw	GGCTCGGCTAGCAGGAGGAAATGTTGTTGAAAAGG
Sporev	GCGACTGCATGCTCACTTTTTCTTTCCTCCG
Y93fw	CCGCGAGCTAGCAGGAGGAGAACAAAATTG
Y93rev	CTCTTGGAAGATGCGCTG
S170fw	AACAGCGCATCTTCCAAGAGGCTTTACAGCCGGAAATGC
S170rev	GCGACTGCATGCTCACTTTTTCTTTCCTCCG
chimera123fw	CGGGCGCCATGGTAAAAACATATCAAAAACTTTTGGCTATGGG
chimera123rev	CGGCGCTCTAGATCACTTTTTCTTTCCTCCG
chimera456fw	CGCCCGCCATGGTGTTGAAAAGGAGAATAGGGTTG
chimera456rev	CGCCCGTCTAGATTATTTCACCGACTCAGTAAGAGC
chimera123his rev	GCGTCTAGATCAGTGATGGTGATGGTGATGCTTTTTCTTTCCTCCGGCTTTTT GCGGC
chimera456his rev	GCGTCTAGATTAGTGATGGTGATGGTGATGTTTCACCGACTCAGTAAGAGC GGCTGTTTTTTTAC
pNZtest1	ACCTGCCCCGTTAGTTGAAGAAG
pNZtest2	GGCTATCAATCAAAGCAACACGTGCTG
pDRtest1	CGTTGCTCGAGGGTAAATG
pDRtest2	CGATCTTTCAGCCGACTC
Y_{TMS12}-S_{TMS345}	
Y169fw	GCGGCCAAGCTTAGGAGGAGAACAAAATTG
Y169rev	CGAGTGTGACGCAATTTCAGGTGTTGAG
S100 fw	CGCTCAACACCTGAAATTGCGTCACACTCGTTCTTATGGTTTGACTTAGGAG
S100 rev	GCGACTGCATGCTCACTTTTTCTTTCCTCCG
Y_{TMS123}-S_{TMS45}	
Y209fw	GCGGCCAAGCTTAGGAGGAGAACAAAATTG
Y209rev	TTGCGGGACAGCGGAATATTTCG
S64 fw	CGCGAAATATTCCGCTGTCCCGCAACAAAATCCGCAAATGGCGATGATG
S64 rev	GCGACTGCATGCTCACTTTTTCTTTCCTCCG
S_{TMS1}-$Y_{TMS2345}$	
S91fw	GGCTCG GTCGAC AGGAGGAAATGTTGTTGAAAAGG
S91rev	CTGCATCGCTTTCGAACTTCTCAG
Y187fw	CTGAGAAGTTCGAAAGCGATGCAGCAGCGCATCTTCCAAGAG
Y187rev	GCCTGCGCTAGCTTATTTCACCGACTCAGTAAGAG
S_{TMS12}-Y_{TMS345}	
S_{166}fw	GGCTCG GTCGAC AGGAGGAAATGTTGTTGAAAAGG
S_{166} rev	GTCAAACCATAAGAAGCTATGCTCTG
Y_{177}fw	CAGAGCATAGCTTCTTATGGTTTGACCAATCTGATATTCTCATGTCCC
Y_{177}rev	GCCTGCGCTAGCTTATTTCACCGACTCAGTAAGAG
S_{TMS123}-Y_{TMS45}	
S_{197}fw	GGCTCG GTCGAC AGGAGGAAATGTTGTTGAAAAGG
S_{197} rev	CTGCGCATTGCCAGCCATCATCAG
Y_{67}fw	CTGATGATGGCTGGCAATGCGCAGCAAAACCCTGCAGCGCAGCAATCC
Y_{67}rev	GCCTGC GCTAGC TTATTTCACCGACTCAGTAAGAG
Y_{P1}-$S_{TMS12345}$	
Y_{54}fw	CCGCGAGCTAGCAGGAGGAGAACAAAATTG -3'
Y_{54}rev	ACCCTTAAGCAGAGCGGAAAACGGTTCG -3'
S_{53}fw	TCCGCTCTGCTTAAGGGTGTAGCGAAATTGACGGGAGATAAC-3'
S_{53}rev	GCGACTGCATGCTCACTTTTTCTTTCCTCCG-3'

S_{P1}-$Y_{TMS12345}$	
S_{52}fw	GGCTCG GTCGAC AGGAGGAAATGTTGTTGAAAAGG-3’
S_{52}rev	ATACGTAATGAGCTCAGACAATGGATATAC-3’
Y_{55}fw	CCATTGTCTGAGCTCATTACGTATGTTGCCGGGCTGTTTCACGGAG-3’
Y_{55}rev	GCCTGCGCTAGCTTATTTCACCGACTCAGTAAGAG-3’